

In the Specification:

Amend specification to read as follows:

Amend the specification on page 4 lines 26-31 through page 5 lines 1-2 , as follows:

C4 One characteristic of "functionality" or "biological functionality" is that the polynucleotide encodes for a S1P receptor; it responds to S1P and optionally also to related phospholipids like DMS 1P or LPA. By "functionality" is meant the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of Ca^{2+} mobilization in response to S1P mediated by the receptor, e.g., EDG8 or a functional fragment thereof, in CHO cell as set forth in the description of Figures 4-9.

Amend the specification on page 7 lines 28-30, as follows:

C5 Figures 1A and 1B (SEQ ID NO:1 and 2): The nucleotide and deduced amino acid sequence of human EDG8. The deduced amino acid sequence (SEQ ID NO:2) is shown below the nucleotide sequence (SEQ ID NO:1) with the nucleotide positions indicated on the left.

Amend the specification on page 8 lines 1-4, as follows:

C6 Figure 2: Phylogenetic tree of the EDG-family of receptors. The phylogenetic tree depicted was derived by the neighbor joining method performed with the GCG program Wisconsin package version 10.1-Unix (Genetic Computer Group (GCG), Madison, Wisconsin.

Amend the specification on page 8 lines 6-13, as follows:

C7 Figures 3A and 3B (SEQ ID NOs. 2-9): Alignment of the amino acid sequence of human EDG8 with the other EDG-family members. The amino acid sequence (amino acids positions 1 through 418) of human EDG8 (accession number AC011461, SEQ ID NO:2) is compared with the EDG1-7 polypeptides (EDG1 (SEQ ID NO:6): accession number

M 31210, EDG2 (SEQ ID NO:3): accession number U 80811, EDG3 (SEQ ID NO:7): accession number X 83864, EDG4 (SEQ ID NO:5): accession number AF 011466, EDG5 (SEQ ID NO:8): accession number AF 034780, EDG6 (SEQ ID NO:9): AJ 000479, EDG7 (SEQ ID NO:4): accession number AF 127138). The approximate boundaries of the seven putative transmembrane domains are boxed. Gaps are introduced to optimize the alignment.

Amend the specification on page 8 lines 14-26, as follows:

Figures 4-9: Mobilization of intracellular Ca^{2+} by S1P (10, 100 and 1000 nM) mediated by the EDG1, 3, 5, 6 and 8 receptor in CHO cells, cotransfected with empty vector DNA as a control or the indicated G-protein α subunits. Figure 4: S1P-induced Ca^{2+} -response in CHO cells transfected with vector DNA alone or the G protein α subunits Gq, G16 and Gqi5. Figures 5-9: S1P-induced Ca^{2+} -response in CHO cells transfected with the indicated EDG-receptor subtypes. Agonist-mediated changes of intracellular Ca^{2+} were measured with the FLIPR using the Ca^{2+} -sensitive dye FLUO4 as described in Experimental procedures. Fluorescence of transfected cells loaded with FLUO4 was recorded before and after addition of S1P, applied in the indicated concentrations. Data are expressed as means of quadruplicate determinations in a single experiment. An additional experiment gave similar results.

Amend the specification on page 8 lines 28-32 through page 9 lines 1-2, as follows:

Figures 10-11: Effects of S1P, LPA and related lysophospholipid mediators on EDG8-mediated increase in intracellular Ca^{2+} . CHO-cells were cotransfected with EDG8 and the G protein α subunits Gqi5 (upper panel) and G16 (lower panel) and rises in $[\text{Ca}^{2+}]$ were recorded with the FLIPR as described in Experimental procedures. The different lipids were applied in concentrations of 10, 100 and 1000 nM, respectively. Data are means of quadruplicate determinations of a representative experiment. Two additional experiments gave similar results.

Amend the specification on page 9 lines 4-8, as follows:

C10 Figure 12: Northern blot analysis of EDG8 in human tissues. Poly(A)+ RNA (1 μ g) from various human tissues (human multiple tissue Northern blots, CLONTECH) was hybridized with probes specific to human EDG8 (upper panel) and β -actin (lower panel) on a nylon membrane. The origin of each RNA is indicated at the top, the molecular mass of standard markers in kilobases (kb) is shown on the left.

Amend the specification on page 9 lines 10-19, as follows:

C11 Figure 13: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of EDG8 in different human endothelial cell lines (HUVECs: human umbilical vein endothelial cells; HCAEC: human coronary artery endothelial cells; HMVEC-L: human microvascular endothelial cells from lung; HPAEC: human pulmonary artery endothelial cells). EDG8-specific transcripts were detected in all endothelial cell lines. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. Amplification with EDG8-specific primers yields a 522 bp EDG8-fragment as indicated by the arrow. The EDG8 plasmid served as a template for the positive control, H_2O was used instead of plasmid DNA as a negative control.

Amend the specification on page 9 lines 21-27, as follows:

C12 Figure 14: PCR analysis of EDG8 primers for specificity of amplification of EDG8 sequences. Primers, specific for the EDG8 sequence, were checked for potential amplification of the related EDG1-7 sequences, using the respective plasmids as templates. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. The EDG8 specific 522 bp band occurred only when EDG8 was used as a template. H_2O was used instead of plasmid DNA as a negative control.

Amend the specification on page 9 lines 29-30, as follows:

C13 Figures 15-17: Experiments were performed according to example 3. Instead of lipids, a lipid library was used.

Amend the specification on page 9 line 32, as follows:

C14 Figures 15-16: Library plates with rat EDG8 (r EDG8) and q15.

Amend the specification on page 10 line 2, as follows:

C15 Figure 15: q15 background.

Amend the specification on page 10 line 4, as follows:

C16 Figure 16: Measurement with rEDG8.

Amend the specification on page 10 line 6, as follows:

C17 Figure 17: Fluorescence change counts.

Amend the specification on page 10 lines 8- 9, as follows:

C18 Figures 18-20: Experiments were performed according to example 3. Instead of Lipids, a lipid library was used.

Amend the specification on page 10 line 10, as follows:

C19 Figures 18-19: Library plates with human EDG8 (hEDG8) and q15.

Amend the specification on page 10 line 12, as follows:

C20 Figure 18: q15 background.

Amend the specification on page 10 line 14, as follows:

C21 Figure 19: Measurement with hEDG8

Amend the specification on page 10 line 16, as follows:

C22 Figure 20: Fluorescence change counts.

Amend the specification on page 10 line 18-31, as follows:

C²³

Figures 21-22: Antagonism of S1P activation of rat and human EDG8. Transiently transfected CHO cells expressing rat EDG8 and G_{q/11} (Figure 21) and HEK 293 cells expressing human EDG8 and G_{q/11} (Figure 22) were incubated with test compounds, namely, 0.1μM Leukotriene B4, 1μM 2-DHLA-PAF (1-O-Hexadecyl-2-O-dihomo-γ-linolenoyl-sn-glycero-3-phosphorylcholine), 1μM C₂ Dihydroceramide, 0.1μM 15(S) HEDE (15(S)-Hydroxyeicosa-11Z,13E-dienoic acid), 1μM PAF C16 (1-O-Hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine), 1μM 16,16 Dimethyl PGE₂ (16,16-Dimethyl-Prostaglandin E₂) 12, 0.1μM (R)-HETE (12(R)-Hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoc acid), 1μM 8-epi-PGF_{2α} (8epi-Prostaglandin F_{2α}) 0.1μM Leukotoxin A ((±) 9,10-EODE) or with solvent buffer for 3 min and then challenged with 1μM S1P (sphingosine 1-phosphate). Peak fluorescence counts of cells preincubated with solvent buffer and then stimulated with 1μM S1P were set 100 %. Fluorescence change counts were recorded with the FLIPR as described in detail in Experimental procedures. Data are means + SE of 2-3 independent experiments.

Amend the specification on page 11 lines 1-11, as follows:

C²⁴

Figures 23-26: Inhibition of S1P mediated intracellular calcium release by suramin and NF023 (8,8`-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphatlenetrisulfonic acid)) in cells transiently cotransfected with human EDG8 and G_{q/11} (Figures 23-24) and rat EDG8 and G_{q/11} (Figures 25-26). Transfected cells were first treated with the indicated concentrations of the inhibitor or solvent buffer for 3 minutes (NF023 and suramin did not show any effect on [Ca²⁺]_i mobilization during the preincubation period). Cells were then stimulated with 1μM S1P and [Ca²⁺]_i measured with the FLIPR as described in the method section. Peak fluorescence counts were normalized and background responses of G_{q/11}-transfected cells were subtracted. S1P-mediated calcium release in the absence of inhibitor was set 100%. Data are means + SE of 4-7 independent experiments.

Amend the specification on page 11 lines 26-31 through page 12 lines 1-7 , as follows:

In particular, the invention relates to an EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which has at least about 90 %, preferably at least about 95 %, most preferred about 98 % or more identity to the amino acid sequence SEQ ID NO. 2 or to a part of SEQ ID NO. 2. In particular the invention relates to an EDG8 polypeptide or a fragment thereof having amino acid sequence SEQ ID NO. 2 or a part thereof. In particular, the invention relates to an polypeptide encoded by SEQ ID NO. 1 or encoded by a polynucleotide that has at least about 90 %, preferably at least about 95 %, most preferred about 98 % or more identity with SEQ ID NO. 1; preferably, such polypeptide has almost the same properties as human EDG 8; e.g. the same biological activity or functionality. One characteristic functionality of human EDG8 is that the polypeptide is a S1P receptor; it responds to S1P and optionally to related phospholipids like dHS1P or LPA as depicted and described in Figures 4-9.

C25

Amend the specification on page 14 lines 15-31, as follows:

It is understood that all nucleic acid molecules encoding EDG8 are also included herein, as long as they encode a polypeptide having the biological activity of human EDG8. By "EDG8 biological activity" is meant that the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of the ability of Ca^{2+} to mobilize as described in Figures 4-9. Such nucleic acid molecules include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, DNA encoding EDG8 may be subjected to site-directed mutagenesis. The nucleotide sequence for EDG8 also includes antisense sequences, and sequences encoding dominant negative forms of EDG8. The invention includes nucleotide sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of EDG8 polypeptide encoded by the nucleotide sequence is functionally unchanged. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:1 are replaced by ribonucleotides A, G, C, and U, respectively.

C26

Amend the specification on page 18 lines 21-29, as follows:

The polynucleotides which hybridize to the above described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of SEQ ID NO:1. For example, such polypeptide could function as a receptor for S1P and related compounds, viz., LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of Ca^{2+} mobilization in response to S1P mediated by the receptor, e.g., EDG8 or a functional fragment thereof, in CHO cell as described in Figures 4-9.

Amend the specification on page 33 lines 8-23, as follows:

As explained above, the invention refers also to a protein encoded by one of the DNA sequences as aforementioned. This protein has activity of a EDG8. Activity of EDG8 is meant the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of Ca^{2+} mobilization as described in Figures 4-9. As described above, further included is production of a protein wherein first a host cell harboring a recombinant vector including a DNA sequence encoding for an amino acid sequence or a polynucleotide sequence for EDG8 is propagated in a suitable growth medium chosen from either media for bacteria or eucaryotic cells depending on the related host cell type. These propagated cells are second harvested by common methods of biochemistry as centrifugation or filtration and processed to obtain crude cell extracts. These cell extracts third are purified subsequently by methods used for protein purification as size exchange chromatography, ion exchange chromatography, affinity chromatography and others to gain the protein of interest (EDG8 activity) separated from other compounds of the cell lysates.

Amend the specification on page 40 lines 1-7, as follows:

C²⁹ Performing a PSI-BLAST search, the various cDNAs and genomic contigs, respectively, for the human EDG1-7 receptors were identified, and an additional genomic hit, highly homologous to human EDG5 (51% homology), termed EDG8. The nucleotide and amino acid sequence of the new putative GPCR are depicted in Figures 1A and 1B. Hydropathy analysis (hydrophobicity plot not shown) suggests a seven transmembrane protein with three alternating extra- and intracellular loops, assumed to be the heptahelix structure common to GPCRs.

Amend the specification on page 40 lines 9-30, as follows:

C³⁰ To shed more light on the relationships involved in the molecular evolution of the EDG-receptor family, a grow tree phylogram was constructed using the neighbor joining method (Genetic Computer Group (GCG), Madison, Wisconsin. (Figure 2) Comparison of amino acid sequences). According to this phylogenetic tree, the human EDG-family can be divided into two distinct groups: EDG1, 3, 5 and 6 belonging to one, EDG2, 4 and 7 belonging to the other group. These two groups are discriminated further by their preference for different lipid ligands: EDG1, 3, 5, 6 are preferentially stimulated by sphingosin 1-phosphate (S1P) (Yatomi et al., J Biochem (Tokyo) 12:969, 1997; Lee et al., Science 279:1552, 1998; Lee et al., J Biol Chem 273:22105, 1998; Ancellin and Hla, J Biol Chem 274:18997, 1999; Yamazaki et al., Biochem Biophys Res Commun 268:583, 2000; Van Brocklyn et al., Blood 95:2624, 2000), EDG2, 4 and 7 by lysophosphatidic acid (LPA) (Hecht et al., 1996; An et al., J Biol Chem 273:7906, 1998; Im et al., Mol Pharmacol 57:753, 2000). The newly identified EDG8 exhibited highest similarity (86.8% amino acid identity) to the rat nrg1-protein (Figure 2), a GPCR recently cloned by EST-expression profiling from a rat PC12 cell library (Glickman et al., Mol Cell Neuroscience 14:141, 1999), which probably represents the rat homologue of human EDG8. In the report of Glickman et al., however, the authors did not address the question of the activating ligand of this receptor. The high similarity between EDG8 and the known sphingosin 1-phosphate (S1P) receptors EDG1, 3 and 5 (48-51%) (Figures 3A and 3B) led to test the hypothesis that EDG8 may be a functional S1P-receptor.

Amend the specification on page 42 lines 1-24, as follows:

Figures 4-9 depict measurement of the intracellular Ca^{2+} concentration, mediated by S1P via the putative S1P receptor EDG8. For sake of comparison, the S1P-receptors EDG1, 3, 5, and 6, which have been reported to mobilize $[\text{Ca}^{2+}]_i$, were included. $[\text{Ca}^{2+}]_i$ were recorded as real time measurements using the Fluorescence plate imaging reader (FLIPR, Molecular Devices). Initially, CHO cells transfected with empty vector DNA were stimulated with different concentrations of S1P (10, 100, 1000 nM). None of the applied S1P concentrations was capable of eliciting significant rises in intracellular Ca^{2+} (Figure 4), suggesting that S1P receptors are not expressed in CHO cells or, if expressed, are unable to signal via the endogeneous $\text{G}\alpha_q$ pathway. To address this issue, the G protein chimera $\text{G}\alpha_{qi5}$, which confers onto Gi coupled receptors the ability to stimulate the Gq pathway, and $\text{G}\alpha_{16}$, which links Gi- and Gs coupled receptors to PLC β and subsequent intracellular Ca^{2+} -mobilization were used. Upon stimulation with S1P, $\text{G}\alpha_{qi5}$ - and $\text{G}\alpha_{16}$ -transfected CHO cells did not give rise to significant increases in $[\text{Ca}^{2+}]_i$ (Figure 4). However, transient transfection of CHO-cells with the cDNAs coding for the EDG1, 3 and 5 receptor conferred S1P-responsiveness to the cells: it was confirmed that EDG1, 3 and 5 mobilize $[\text{Ca}^{2+}]_i$ in response to S1P (Figures 5, 6, 7) (Kon et al., J Biol Chem 274:23940, 1999). As already known for a large number of Gq-coupled receptors, coexpression of $\text{G}\alpha_q$ augments the EDG1 and 5-mediated Ca^{2+} -response as compared with the Ca^{2+} signal induced by stimulation of endogeneous $\text{G}\alpha_q$. In case of EDG3, additional exogeneously added $\text{G}\alpha_q$ did not further improve the signal intensity. These results are in agreement with the findings reported by Kon et al. (J Biol Chem 274:23940, 1999), who showed that the EDG3-subtype causes the most robust enhancement of intracellular Ca^{2+} .

C31

Amend the specification on page 42 lines 26-29 through page 43 lines 1-8, as follows:

In case of EDG6, Yamazaki et al. (Biochem Biophys Res Commun 268:583, 2000) obtained an S1P-induced mobilization of $[Ca^{2+}]_i$ but in this study, investigators failed to detect a significant Ca^{2+} increase above basal levels in the absence of any cotransfected G-protein α subunit Figure 8. The reason for this discrepancy could be the cellular background (CHO cells in this study vs. K562 cells in Yamazaki et al., Biochem Biophys Res Commun 268:583, 2000), as they reported a pertussis toxin (PTX)-sensitive Ca^{2+} -response, indicating the involvement of Gi-type G-proteins. In this case the Ca^{2+} signal would be elicited by β , released from activated $G\alpha_i\beta$ heterotrimers. The $G\alpha_i$ -induced Ca^{2+} signals are known to be much smaller in intensity as compared with the Ca^{2+} signals induced by bona-fide Gq-linked receptors (Kostenis et al., J Biol Chem 272:19107, 1997). It may be that detection of such $[Ca^{2+}]_i$ concentrations is beyond the sensitivity of the FLIPR system.

Amend the specification on page 43 lines 10-23, as follows:

EDG8 did not release $[Ca^{2+}]_i$ when stimulated with S1P (10, 100, and 1000 nM) Figure 9, but gained the ability to mobilize Ca^{2+} upon cotransfection with $G\alpha_{16}$, a G-protein α subunit, known to couple GPCRs from different functional classes to the Gq-PLC β pathway or $G\alpha_{qi5}$, a mutant G-protein α subunit that confers onto Gi-linked receptors the ability to stimulate Gq (Conklin et al., 1993). These results show that EDG8 is a functional receptor for S1P and that EDG8-induced Ca^{2+} responses are due to a non-Gq pathway, probably the activation of phospholipase C $\beta2$ by β subunits of the Gi proteins. Furthermore, these results provide additional evidence that the S1P-preferring EDG-receptors couple differentially to the Gq and Gi pathways: EDG3 is the most potent Ca^{2+} -mobilizing receptor and overexpression of $G\alpha_q$ does not further improve Ca^{2+} signalling; EDG1 and 5 induce moderate Ca^{2+} -increases, that can be significantly improved by cotransfection of $G\alpha_q$ or a chimeric $G\alpha_{qi5}$ protein; EDG8-mediated Ca^{2+} -responses require cotransfection of $G\alpha_{qi5}$ or $G\alpha_{16}$.

Amend the specification on page 43 lines 25-29 through page 44 lines 1-11, as follows:

To check whether the EDG8 receptor also reacts to related lysophospholipid mediators, the inventors examined the abilities of lysophosphatidic acid (LPA), dihydrosphingosin 1-phosphate (dHS1P), sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) to increase intracellular Ca^{2+} in CHO cells transiently transfected with the EDG8 receptor and the G-protein α subunits $\text{G}\alpha_{16}$ and $\text{G}\alpha_{q/11}$ (Figures 10 and 11). Besides S1P, which was the most potent activator of EDG8, LPA and dHS1P evoked $[\text{Ca}^{2+}]_i$ increases in concentrations of 100 and 1000 nM. SPC and LPC, respectively, failed to generate any significant response in concentrations up to 1 μM . These data show that EDG8 is a S1P preferring receptor, but also responds to related phospholipids like dHS1P or LPA, as has also been reported for EDG1, which is a high affinity receptor for S1P and a low affinity receptor for LPA (Lee et al., J Biol Chem 273:22105, 1998). Therefore, EDG8 receptor has the characteristic functionality to respond to S1P and related phospholipids like DMS 1P or LPA. The response to S1P and other related phospholipides can for example be determined as described in Example 3. Cells containing the respective $\text{G}\alpha$ can be obtained as described in Example 2.

Amend the specification on page 44 lines 13-27, as follows:

Next, the expression pattern of the EDG8 gene in human tissues was investigated by Northern blot analysis Figure 12. Tissues positive for EDG8 RNA were skeletal muscle, heart and kidney, lower abundance of RNA was seen in liver and placenta, no signal was detected in brain, thymus, spleen, lung and peripheral blood leukocytes. In all tissues a single RNA transcript of 5.5 kb was observed after hybridization with a DIG-labeled EDG8 antisense RNA probe. EDG8 exhibits highest similarity to the rat nrg1-GPCR (Glickman et al., Mol Cell Neuroscience 14:141, 1999) with an amino acid identity of 86.8% Figure 2 suggesting that it may be the human homologue of the rat nrg1 protein. However, the expression pattern of human EDG8 is quite different from the rat nrg1-receptor, which is found almost exclusively in brain (Glickman et al., Mol

Cell Neuroscience 14:141, 1999). This finding suggests that EDG8 may represent a closely related but entirely different receptor from nrg1, rather than the human homologue. Never the less, it does not rule out the possibility that EDG8 and nrg1 are homologues with entirely different, species-dependent expression patterns.

Amend the specification on page 45 lines 1-12, as follows:

Exposed to fluid shear stress as an upregulated gene it is reasonable to assume that EDG receptors play an important role in the regulation of endothelial function. Therefore, the presence of EDG8 transcripts in several human endothelial cell lines was analyzed. RT-PCR analysis of human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), human microvascular endothelial cells of the lung (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) revealed EDG8 expression in all cell lines tested (Figure 13). In Figure 14 it is shown that EDG8 specific primers indeed solely amplify EDG8 sequences and none of the related EDG1-7 sequences. These findings suggest that the presence of EDG8 in different peripheral organs may be due to its localization in endothelial cells; it does not rule out, however, that EDG8 transcripts occur in cell types other than endothelial cells.